Detection of phytoplasma in *Allocasuarina fraseriana* and *Acacia saligna* in Kings Park

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Abstract

Phytoplasma-like symptoms which included bunchy growth, witches' broom and 'little leaf' were observed in *Allocasurina fraseriana* (Western Sheoak, Casuarina) and *Acacia saligna* (Acacia, Orange Wattle) trees in Kings Park and Botanic Garden. The affected trees were in a poor state of health or appeared to be dying. Many trees of other species exhibited similar symptoms. A sensitive, diagnostic method based on the polymerase chain reaction (PCR) was used to detect the presence of phytoplasma in symptomatic and asymptomatic leaf and root samples from the trees. The amplified PCR products were sequenced and the sequences submitted to Genbank as Casuarina (accession number EF474451) and Acacia (accession number EF474452). Sequence analysis indicated that the phytoplasma detected in plants belonged to the 16Sr II group and was closely related to *Candidatus* phytoplasma aurantifolia. Phytoplasma-associated disease has therefore been confirmed for the first time in casuarina and acacia trees in Western Australia.

Keywords: Western Sheoak, *Casuarina*, Acacia, Orange wattle, Common Wattle, *Candidatus* phytoplasma aurantifolia

Introduction

Phytoplasmas are phloem-limited plant pathogens of the class *Mollicutes*. Symptoms of phytoplasma-associated disease vary between host plant species and also the types of phytoplasma. Infection may be characterised by stunted growth, reduced leaf size, little leaf, witches' broom, leaf deformation, hardened stems, leaf and stem discolouration, sterility, phyllody, virescence or gigantism, dieback, and big bud (Davis *et al.* 1997; Lee & Gundersen-Rindal 2000). In recent studies phytoplasma-associated diseases have been identified in Western Australia (WA) affecting several host species including chickpea, tomato, snakebean, paddymelon, sweet potato, clovers and Paulownia trees (Davis *et al.* 1997; Bayliss *et al.* 2005; Saqib *et al.* 2005; 2006a; 2006b; Tiaro *et al.* 2006), although the identity of plant species that act as reservoirs for phytoplasmas that affect crop species is not known.

A. fraseriana (Western Sheoak or Casuarina) is native to WA and grows in the region between Perth and Albany. *A. saligna* (Orange wattle, Common wattle) is also native to the southwest region of WA. Many of the casuarina and acacia trees in Kings Park and Botanic Gardens showed symptoms of witches' broom, stunted growth, dying back of branches and small leaves. Confirmation of the presence of phytoplasma disease associated with these trees in WA is lacking although a phytoplasma-associated disease was reported in *Allocasurina muelleriana* in South Australia (Gibb *et al.* 2003).

Phytoplasma in plants can be identified using the polymerase chain reaction (PCR) by amplification of the 16S rRNA genes with RFLP analysis of the product to differentiate 'clades', and nested PCR can be used to increase the sensitivity of the assay (Streten & Gibb 2006). The differences in sequences of 16S rRNA genes helps to differentiate members of the phytoplasma clade into at least 15 groups (Lee & Gundersen-Rindal 2000; IRPCM Phytoplasma/Spiroplasma Working Team–Phytoplasma Taxonomy Group 2004). Nested PCR and subsequent DNA sequence analysis has been used to confirm the presence of phytoplasma disease in symptomatic casuarina and acacia trees in Kings Park and Botanic Garden, Perth.

Methods

Leaf and root samples were collected in Kings Park and Botanic Garden from both symptomatic and asymptomatic trees of *A. saligna* and *A. fraseriana* in September 2006 with further samples collected in July 2007. Because uninfected control plants could not be found in Kings Park, further leaf and root samples of the two species were collected from the Banksia Reserve, Murdoch University, South Street campus in August 2007. The affected plants were selected based on the presence of symptoms such as witches' broom, stunted growth, little leaf and controls samples were collected that had no visible symptoms. Root and leaf samples from two symptomatic *A. saligna* trees and four symptomatic *A. fraseriana* trees from Kings Park were analysed. The *A. fraseriana* and *A. saligna* trees with severe witches' broom structures had roots that appeared to be growing normally. Similarly root and leaf samples from two asymptomatic trees each of *A. saligna* and *A. fraseriana* were collected from Kings Park and Banksia Reserve at Murdoch University as negative controls. All

root samples were washed twice with deionised water to remove adhering soil and debris, followed by rinsing with 100% ethanol then 70% ethanol. The samples were stored overnight at 4°C in plastic bags before DNA extraction.

The hot cetyltrimethylammonium bromide (CTAB) method was used to extract DNA from 1g of the leaf and root tissue samples separately (Dellaporta *et al.* 1983; Zhang *et al.* 1998). The first round of PCR was done using primers P1 and P7 followed by a second round using internal primers R16mR1 and R16mF2 (Deng & Hiruki 1991). The PCR conditions were as described in Saqib *et al.* 2006b. The nested PCR products (~1400bp) were then analysed by electrophoresis on a 1% agarose gel. A previously cloned 16S rRNA gene of Sweet Potato Little Leaf (SPLL) phytoplasma isolated from snakebean GenBank (accession no DQ375777) was used as a PCR positive control (Saqib *et al.* 2006b).

The ~1400bp PCR product was sequenced directly from both ends using Big Dye Terminator 3.1 (Perkin-Elmer, Foster City, CA), with an Applied Biosystems 3730 DNA capillary sequencer. The nested PCR products were sequenced using 3.2 picomole of primer R16mR1 and R16mF2. Two different PCR products from each sample of leaves and roots were sequenced to check for the possibility of mixed infection with different phytoplasmas and to prevent errors in editing. The 16S rRNA gene sequences were analysed using BioEdit™ version 7.0.5.3 software.

Results

The symptoms of disease-affected *A. fraseriana* and *A. saligna* trees were consistent wherever they occurred in Kings Park. The majority of symptomatic trees appeared to be dying or were already dead. The most common symptoms observed were witches' broom, stunted growth, little leaf and dying back of the branches (Fig. 1 & 2). Nested PCR analysis of the *A. fraseriana* and *A. saligna* samples confirmed the presence of phytoplasma. The phytoplasma infections were identified in all symptomatic leaf and some root samples (Fig. 3). One root sample from symptomatic *A. saligna* and three root samples from symptomatic *A. fraseriana* were negative for phytoplasma infection whilst leaf samples from the same plants were positive for phytoplasma-associated disease. All of the samples from asymptomatic plants of both species collected specifically as negative controls in Kings Park were also found to contain phytoplasmas (Fig. 4). PCR controls and absence of diagnostic bands in source samples showed that this was not a result of experimental contamination. As a result of this observation asymptomatic samples of *A. fraseriana* and *A. saligna* from Murdoch University were also analysed. In this case clear negative controls were obtained of both species, except in one root sample from *A. fraseriana* (Fig. 5).

The 1400 bp regions between primers R16mR1 and R16mF2 (corresponding to the16S rRNA gene) from both *A. fraseriana* and *A. saligna* plants were sequenced. Sequences from *A. fraseriana* and *A. saligna* root and leaf samples were 100% homologous. Partial sequence data from symptomatic and asymptomatic *A. fraseriana*

Figure 1. Diseased *A. fraseriana* tree in Kings Park (September, 2006). Many leaves and branches were dead or dying, but the bunchy growths with little leaves and multiple stunted shoots are clearly visible.

Figure 2. Diseased *A. saligna* tree in Kings Park with phytoplasma-like symptoms such as little leaf, stunted bushy growth, and witches' broom (September, 2006).

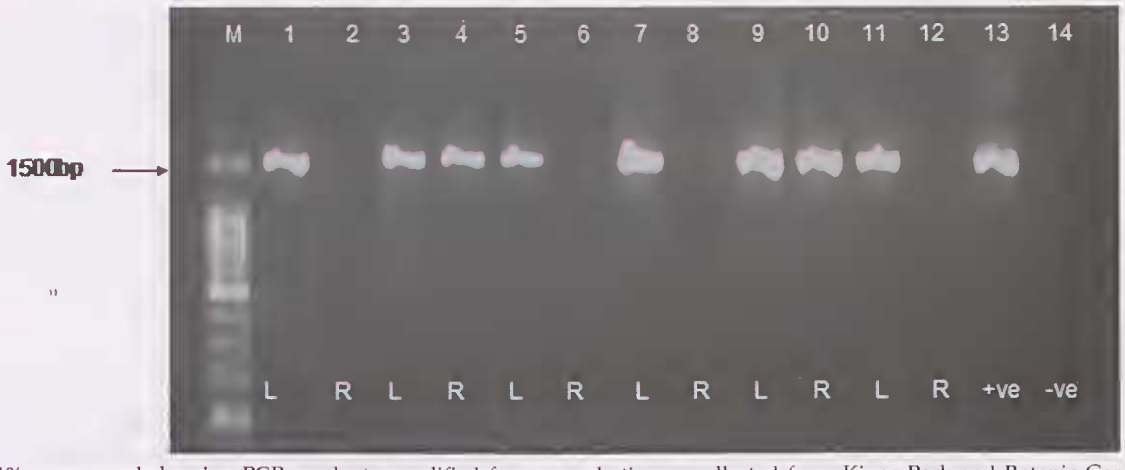

Figure 3. 1% agarose gel showing PCR products amplified from sample tissues collected from Kings Park and Botanic Garden. M = 100bp ladder (Promega). L = Leaf tissue. R = Root tissue. Lanes 1-4, *A. saligna*: Lanes 5-12, *A. fraseriana*: Lane 13, PCR positive control (SPLL): Lane 14, PCR negative control.

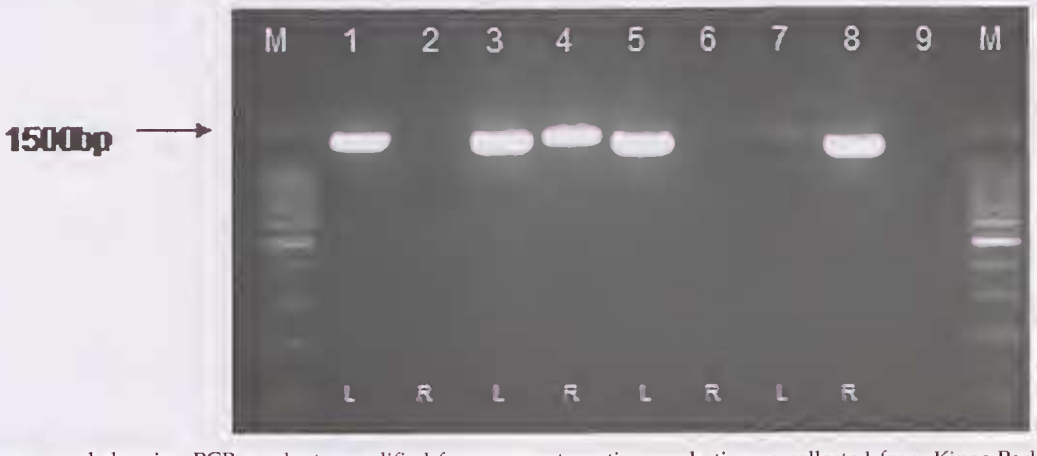

Figure 4. 1% agarose gel showing PCR products amplified from asymptomatic sample tissues collected from Kings Park and Botanic Garden. M = 100bp ladder (Promega). L = Leaf tissue. R = Root tissue. Lanes 1-4, *A. saligna*: Lanes 5-8, *A. fraseriana*: Lane 9, PCR negative control.

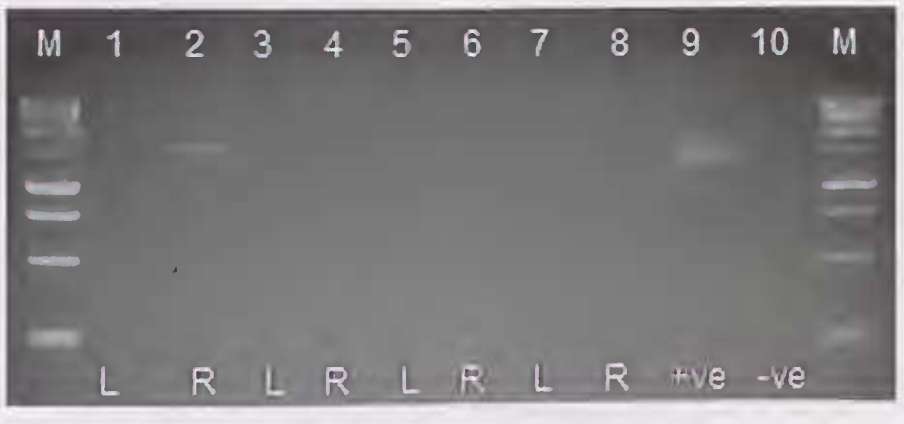

Figure 5. 1% agarose gel showing PCR products amplified from asymptomatic tree samples collected from the Banksia Reserve, Murdoch University. M = 1Kb ladder (Promega). L = Leaf tissue. R = Root tissue. Lanes 1-4, *A. fraseriana*: Lanes 5-8, *A. saligna*: Lane 9, PCR positive control: Lane 10, PCR negative control.

roots and leaves were reported as one sequence and 1368 bases were submitted in GenBank (accession no EF474451). Similarly partial sequences from *A. saligna* were compared and combined as one sequence and 1362 bases were submitted to GenBank (accession no EF474452). Partial sequence data of 1353 bases from asymptomatic *A. fraseriana* roots from the Banksia Reserve, Murdoch University were also submitted in GenBank (accession no EU095022). A BLASTN search of the NCBI (databasehttp://www.ncbi.nlm.nih.gov/BLAST/Blast) was carried out to identify homology with other phytoplasma sequences. The sequences from *A. fraseriana* and *A. saligna* were 100% homologous to the 16S rRNA gene of Sweet Potato Little Leaf (SPLL) GenBank (accession no AJ289193) belonging to *Candidatus* Phytoplasma aurantifolia (16Sr II, strain V4).

Discussion

Many individuals of *A. fraseriana* and *A. saligna* in King's Park and Botanic Garden appeared unhealthy and exhibited symptoms of witches' broom. These two tree species are prominent in the park and their disfigurement detracts from its aesthetic value. This study was undertaken to seek to identify if phytoplasma disease was an underlying cause of these symptoms. The results of analysis of rRNA genes from DNA extracted from affected tree samples showed the presence of phytoplasmas in all symptomatic trees sampled. Furthermore, the asymptomatic plants collected specifically as negative controls also tested positive either in leaf or root samples or in both. This suggests that the majority of these species of trees in Kings Park are affected with phytoplasma. To obtain clear negative control results, it was necessary to analyse additional asymptomatic samples from a separate site (Murdoch University campus), and even at this site one plant of *A. fraseriana* showed presence of phytoplasma. Based on the sequences of the amplified DNA the phytoplasma present was identified as identical to that of Sweet Potato Little Leaf which belongs to *Candidatus* Phytoplasma aurantifolia (16Sr II). To our knowledge this is the first report of phytoplasma affecting casuarina and acacia trees in WA.

It is possible that other trees and shrubs that display similar symptoms may also be affected by phytoplasmas. It is also possible that other factors, such as lowering of the water table or infection with other pathogens could contribute to the diseased state of the trees in Kings Park. Although phytoplasma-associated disease has been identified in various plant hosts in WA (Bayliss *et al.* 2005; Saqib *et al.* 2006b; Tiaro *et al.* 2006) there is very little information on the extent of phytoplasma infection on other native species or insect vectors and the mode of transmission of phytoplasmas. The insects which have been shown to be vectors of phytoplasma-associated disease (Pilkington *et al.* 2004) and which are also present in WA include leafhoppers (*Membracoidea*), plant hoppers (*Fulgoroidea*) and psyllids (*Psylloidea*).

There are a number of reports of phytoplasma disease in WA in a range of cultivated and native plants (Doepal 1964; Goss 1964; Padovan *et al.* 2000; Saqib *et al.* 2005; 2006a), and it is likely that native plants act as reservoirs of phytoplasmas, from which infection of commercial plants can originate (Saqib *et al.* 2005; Bayliss *et al.* 2005). Plants that appear to be affected by phytoplasma can be found readily in most regions of WA including the Kimberley (Saqib *et al.* 2005), the Southwest (Bayliss *et al.* 2005; Saqib *et al.* 2006a) and Gascoyne region (Saqib *et al.* unpublished). The occurrence of phytoplasma disease in WA remains largely unmonitored and in general this disease has been ignored, so that there is little effort specifically to prevent spread of phytoplasma disease either to native bushland or to crop plants from native reservoirs. This is a subject which requires further research, particularly on vectors and the mode of transmission, understanding the host-pathogen interaction and additional factors that contribute to the diseased state.

Kings Park is culturally significant to Perth, as well as being a valuable source of tourism revenue to the state. To preserve its attractiveness and minimise damage to trees associated with phytoplasma disease, management action, such as control of insect vectors and removal of affected trees could be taken to prevent further spread.

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